Running Title: Anti-μ fails to induce proliferation in M167 $\mu\kappa$ B cells

B Cells From M167 $\mu\kappa$ Transgenic Mice Fail to Proliferate Following Stimulation With Soluble Anti-Immunoglobulin Antibodies: A Model for Antigen-Induced B Cell Anergy [1,2]

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[2]Abbreviations: PC, phosphocholine; PC-KLH, phosphocholine conjugated to keyhole limpet hemocyanin; sIgD, surface IgD; sIgM, surface IgM; TG, transgenic mice; TG^+, transgene positive; TG^-, transgene negative; IL-4, Interlukin 4.

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ABSTRACT

The transgenic mouse strain 207-4, carries μ^a + κ transgenes ligated to the anti-phosphocholine (PC) V_H1 and $V_\kappa24$ variable region genes from the MOPC-167 myeloma. Although B cells from mice carrying these transgenes respond both in vivo and in vitro to thymus dependent antigens, they failed to proliferate in response to soluble goat anti-μ antibody or other soluble anti-Ig reagents. On the other hand, B cells from the Sp6 $\mu\kappa$ anti-TNP transgenic mouse line proliferated normally after stimulation with soluble anti-μ. However, the 207-4 anti-PC transgene positive (TG^+) splenic B cells proliferated when stimulated with anti-μ, anti-idiotype, anti-allotype, or PC-conjugated to Sepharose beads. TG^+ B cells were also induced to proliferate when stimulated with anti-Lyb-2; thus, their defect may be restricted to signaling through sIgM. The lack of response to soluble anti-μ could not be reversed by addition of IL-4, by removal of T cells, by addition of anti-Fc receptor antibody, or by stimulation with $F(ab')_2$ anti-μ. Thus, the failure to proliferate was not due to active T cell suppression or FcR mediated inhibition. In mixed cultures of TG^+ and transgene negative (TG^-) spleen cells, the TG^- cells were able to proliferate normally to soluble anti-μ, indicating that suppressive factors were not involved in the unresponsiveness of the TG^+ anti-PC-specific B cells. These studies suggest that B cells in the 207-4 anti-PC transgenic mice exhibit a defect in activation through their sIgM receptors, and this unresponsiveness may reflect a form of antigen-induced tolerance.

INTRODUCTION

Membrane IgM (sIgM) is a receptor for mitogenesis in B lymphocytes, inasmuch as heterologous and monoclonal anti-μ specific antibodies are able to induce proliferation in B cells (1-3). The role that sIgM and sIgD play in the activation and/or tolerance of B cells has been the subject of many studies which have attempted to elucidate the biochemical mechanisms involved in the cascade of events that lead either to activation and antibody secretion or to tolerance of the stimulated B cells. The recent development of transgenic mice (TG) bearing rearranged Ig genes (4-8), the products of which are expressed as antigen-specific receptors on virtually every B cell, provide new opportunities to address the mechanisms and biochemical pathways involved in antigen induced B-cell activation and tolerance.

The 207-4 $\mu\kappa$ anti-PC transgenic mouse line produced by Storb et al.(5) expresses the transgene encoded M167-idiotype (id) on more than 97% of its B cells (6,9). These B cells respond normally both in vivo (6) and in vitro (10) to the thymus dependent antigen, PC-KLH, and thus represent a unique source of "naive" antigen-specific, Id^+ B cells in which to analyze the mechanisms of antigen-driven or anti-Id-driven differentiation. We have recently shown that the B cells in these mice are clonally deleted in an antigen-specific, receptor-mediated manner when the M167 $\mu\kappa$ anti-PC transgenes are co-expressed in the presence of the xid gene (11). Furthermore, in M167 μ-only transgenic mice, M167-Id^+ B cells, which arise by association of the M167 μ-transgene with an endogenous $V_\kappa 24$ light (L) chain, are expanded 100 to 500 fold over the number expected from random expression of L-chain genes (9). This selective expansion of M167-id^+ B cells also appears to be an antigen-driven, receptor-mediated process. Thus, B cells having the same antigen

specificity appear to be either clonally deleted or clonally expanded depending on the X-chromosome phenotype of the mouse in which they develop.

The B cells that develop in the 207-4 $\mu\kappa$ transgenic mice differ from those of normal mice in that, a) they express high levels of the transgene-encoded product on their surface, b) they express no sIgD, and c) endogenous encoded IgM is expressed on less than 20% of these cells (6). This cell surface phenotype is similar to that of immature B cells that have recently emerged from the bone marrow (12). B cells exhibiting this phenotype are more susceptible to tolerance induction than mature sIgM:sIgD positive B cells (13). In the studies presented in this manuscript, we have analyzed the B cells from the 207-4 transgenic mice for their ability to respond to anti-Ig signals that induce proliferation in normal B cells. The results of these studies revealed a defect in the ability of B cells from 207-4 mice to proliferate in response to soluble anti-Ig-antibodies even though they proliferate in response to the same antibodies conjugated to Sepharose beads. Since this proliferative defect was not observed in the B cells from the $\mu\kappa$ anti-TNP Sp6 transgenic mouse line or when the 207-4 B cells were stimulated through other mitogenic receptors, our results may indicate a selective tolerance mechanism in the PC-specific B cells which results from a previous encounter with autologous or environmental PC during their early development.

MATERIALS AND METHODS

Mice. Transgenic mice carrying the MOPC-167 (M167) mu (μ) plus kappa (κ) transgenes (line 207-4, designation Tg(Igh+Igk)Bril2), were obtained from Dr. U. Storb (Dept. Mol. Genet. & Cell Biol., Univ. Chicago, Chicago, IL) through Dr. R. L. Brinster (School of Veterinary Med., Univ. Pennsylvania, Philadelphia, PA.) and have been described previously (5). These mice are

maintained in our breeding colony by backcrossing transgene positive (TG^+) male to C57BL/6 female mice. The Sp6 anti-TNP transgenic mice produced by Rusconi and Köhler (14) and characterized by Lamers et al. (15) were obtained from Dr. R. Hodes, NIH, Bethesda, NIH, Bethesda, MD. The Sp6 $\mu^a + \kappa$ transgenes have been backcrossed onto a C57BL/6 background. These mice express the IgM^a transgene on all their B cells and co-express endogenous IgM^b and IgD on 10-20 % of the B cells (15; Kenny, unpublished). The M167 $\mu\kappa$ (207-4) mice do not express IgD (6). The transgene encoded IgM^a is expressed at the same intensity on both Sp6 and 207-4 B cells (Kenny, J.J., unpublished data). The progeny of both Sp6 and M167 transgenic mice are typed for the presence of the transgene by ELISA analysis of antibodies bearing the IgM^a allotype (μ^a) (16). BALB/cByJ, C57BL/6J, and DBA/2J mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antibodies. Affinity purified goat anti-μ, goat anti-κ, and $F(ab')_2$ fragments of goat anti-μ antibodies were prepared as described previously (2). Myeloma proteins MOPC104E (IgM,λ) and C.BPC112 (IgM,κ) were produced as ascites from myeloma cell lines provided by Dr. Michael Potter, NCI, NIH, through NCI contract no. CB05596-17 maintained with Hazelton Laboratories America, Inc., Rockville, MD. The mouse IgM mAb and myeloma proteins were purified from ascitic fluid by preparative centrifugation at 147,000 x G for 16 hr., followed by chromatography on a Sepharose 6B column in a 0.01 M borate buffered saline, pH 8.4.

AF6-78.25 (17), a mouse IgG_1,κ mAb specific for the b-allotype of mouse IgM, and DS1 (16) an IgG1,κ mAb specific for the a-allotype of mouse IgM, were purified from ascites by precipitation with 50% saturated ammonium sulfate and passaged through a DEAE-Sephacel (Sigma Chemical Co., St. Louis, MO) column in

0.01 M Tris buffer pH 8.0. Anti-Lyb-2 was produced from ascites as previously described (18).

Cell culture and assay for [methyl-^{3}H] thymidine ([^{3}H]TdR) incorporation. Spleen cells were cultured at 3 x 10^5 cells in flat-bottom microtiter plates (Cluster 96, Costar, Cambridge, MA) in 0.2 ml of MEM containing 10% fetal calf serum, 16 mM Hepes buffer, 5 x 10^{-5} M 2-mercaptoethanol and appropriate concentrations of soluble anti-Ig or anti-Ig, anti-Id, or anti-allotype coupled to Sepharose 4B, as previously described (2). After 48 h, DNA synthesis was measured by a 16-18 h pulse of 1 μCi of 2 mCi/mM ^{3}H-TdR (Dupont NEN, Wilmington, DE). Cell cultures were harvested onto glass fiber filters and counted in a β-scintillation counter. Results are expressed as the geometric mean of triplicate cultures (2).

Flow cytometric analysis. Spleens were gently teased into single cell suspensions in HBSS without phenol red (GIBCO BRL, Grand Island, NY). Erythrocytes were removed by resuspending cells in 5 ml of ammonium chloride solution (M.A. Bioproducts, Columbia, MD) for 1 min and then washing 3 times in HBSS containing 5% FCS (HyClone Laboratories, Inc., Logan, UT) and 0.1% NaN_3 (HBSS + FCS). One-hundred μl aliquots (10^6 cells) were prepared in 5 ml test tubes and 10 μl of FITC- or biotin-conjugated antibody (100μg/ml) in HBSS + FCS was added. The cells were incubated for 30 min on ice and then washed twice in HBSS + FCS. Cells stained with a biotin-conjugated antibody were further reacted with phycoerythrin (PE)-conjugated Streptavidin (Fisher Biotechnology, Silver Spring, MD) for 15 min at 4°C and washed with HBSS + FCS. The cells were then analyzed by flow microfluorimetry as previously described (9).

RESULTS

Unresponsiveness of 207-4 transgenic spleen cells to stimulation by anti-Ig. Spleen cells from one TG^+ and one TG^- mouse were cultured with optimal stimulatory concentrations of soluble anti-μ, or Sepharose-conjugated anti-μ, anti-μ^a, anti-μ^b, or anti-κ antibodies, and after 48 h incubation, cultures were analyzed for proliferation by ^{3}H-TdR uptake. As shown in Table I, spleen cells from the TG^+ mouse were unresponsive to soluble goat anti-μ while the TG^- cultures were stimulated 22-fold over the medium control. TG^+ B cells also failed to respond to treatment with soluble anti-V_H1-id and anti-M167id (28-5-15) at doses up to 200 μg/ml (data not shown). In contrast, the TG^+ spleen cells gave a 15-fold higher response than the medium control after stimulation with the same preparation of goat anti-μ conjugated onto Sepharose beads. TG^+ cultures also responded to goat anti-κ conjugated-Sepharose (Table I), anti-V_H1-id and anti-M167id conjugated to Sepharose (Table II), and LPS (Table I). However, the responses to these mitogens were lower than those given by the TG^- spleen cultures, and the lower responses were probably due to lower concentrations of B cells in the TG^+ cultures, as discussed below.

The data in Table I also demonstrate that the TG^+ and BALB/c spleen cells, but not the TG^- or C57BL/6J cells, responded to anti-μ^a-allotype mAb conjugated to Sepharose. This was expected, since the M167 H-chain transgene encodes the μ^a allotype and all B cells in these mice express both the M167-Id and the μ^a-allotype markers on their surface (6,9). As expected, the anti-μ^b-allotype mAb conjugated to Sepharose was mitogenic for the TG^- and μ^b-allotype control C57BL/6 spleen cell cultures, but not for the TG^+ or BALB/c cells. Less than 20% of the B cells in the TG^+ mice coexpress endogenous μ^b H-chains on their surface (6). This may represent too few cells for effective stimulation in our culture system, or alternatively, the low density of μ^b

chains on the surface may not permit effective signalling. Other spleen cell preparations from 207-4 TG^+ mice have exhibited low levels of proliferation following stimulation with anti-μ^b-conjugated-Sepharose (data not shown). Overall, these results suggest that the transgene-encoded sIgM receptor on 207-4 B cells is capable of transducing mitogenic signals when stimulated by Sepharose conjugated anti-Ig, but not soluble anti-Ig.

The inability of the TG^+ spleen cells to proliferate to soluble anti-μ was reconfirmed in 9 additional experiments in which a total of 28 TG^+ mice were individually analyzed for their response to soluble and bead-conjugated anti-μ. The results in Figure 1A demonstrate that spleen cells from TG^+ mice 14 to 60 wk of age were unresponsive to soluble anti-μ. The data in Figure 1B show that these same TG^+ spleen cells responded at all ages, when stimulated by Sepharose-conjugated anti-μ, although the responses were found to increase with the age of the donor. The increased responsiveness with age is probably due to the corresponding increase in total number of B cells present in older mice (Fig. 1C), since B cells from young mice proliferate as well as those from old mice, following anti-Thy1.2 plus C′ treatment and normalization of the number of B cells placed in culture (Table II). The data in Figure 1C also demonstrate that the percent B cells in TG^+ spleens is generally less than that of the TG^- littermates.

To show that the lack of responsiveness of TG^+ spleen cells was not due to a difference in optimum dose of soluble anti-μ required for stimulation of these cells and/or a difference in the kinetics of their response, spleen cells from TG^+ and TG^- mice were cultured in various concentrations of soluble anti-μ and the cells pulsed with ^{3}H-TdR at different times after stimulation. The TG^+ cells did not respond at any dose, while the TG^- cultures responded at

all doses of anti-μ tested, the TG^- cells continued to proliferate for 96 h, whereas, the TG^+ cells did not proliferate during this same 4 day time period (data not shown).

Inability to show suppression by T cells, factors, or Fc receptor mechanisms. The unresponsiveness of TG^+ B cells to soluble anti-Ig could possibly be due to: 1) FcR mediated inhibition; 2) T-cell suppression; 3) suppressive factors; 4) sIgM receptor modulation; or 5) receptor-mediated cell death. It has been demonstrated that anti-μ-induced activation can be inhibited by the Fc portion of the antibody molecule acting through the B-cell FcR (19). To investigate FcR-mediated inhibition as a possible cause of the unresponsiveness in TG^+ B cells, the spleen cells from TG^+ and TG^- mice were: 1) cultured with soluble goat anti-μ in the presence of various concentrations of purified 2.4G2 anti-FcR mAb (20); and, 2) stimulated with goat $F(ab')_2$ anti-μ. The data in Table III demonstrate that anti-FcR antibody had no effect on the response of the TG^+ spleen cells or the control C57BL/6J spleen cells to soluble anti-μ, while the response of the TG^- spleens cells was enhanced an average of 1.7-fold in the presence of anti-FcR. Stimulation of TG^+ and TG^- spleen cells by $F(ab')_2$ anti-μ resulted in an increase of only 6300 cpm (at the highest concentration) in the proliferation of the TG^+ spleen cells, while the proliferation of TG^- spleen cells was enhanced by 29,000 cpm over the response obtained with an equal molar concentration of intact anti-μ antibody (Table IV). The low response by TG^+ spleen cells at the highest concentration of $F(ab')_2$ anti-μ was still three times lower than the response of the same cells to anti-μ-conjugated Sepharose and 10 times lower than the response to anti-V_B1-Id conjugated beads (data not shown). The low response at the highest concentration of $F(ab')_2$ anti-μ may be due to the stimulation of endogenous B

cells in the cultures. Nevertheless, from these FcR blocking and $F(ab')_2$ stimulation experiments, it is evident that FcR-mediated inhibition is not the primary reason for the lack of anti-μ-induced responses in TG^+ B cells.

As shown in Table II, the removal of T cells by treatment with anti-Thy 1.2 + C' also had no effect on the inability of the TG^+ B cells to respond to anti-μ. Since the anti-Thy + C' treatment essentially eliminated the Con A responsive T cells, the presence of anti-μ-specific or non-specific suppressor T cells in the TG^+ spleen is unlikely. It was also possible that suppressor factors produced by the TG^+ spleen cells were responsible for the failure of TG^+ spleen cells to proliferate following anti-μ treatment. This was also ruled out by the data shown in Table V. When TG^+ and TG^- spleen cells were co-cultured in the presence of anti-μ, there was no suppression of the TG^- spleen cell response to soluble anti-μ; in fact, the addition of TG^+ spleen cells to the TG^- cells resulted in a higher response than that obtained with irradiated filler cells.

Anti-Lyb-2-induced proliferation of B cells from $TG^{\pm}$ mice. To elucidate whether the unresponsiveness of TG^+ B cells to soluble anti-μ was limited to stimulation through the sIgM receptor, spleen cells from (207-4 x DBA/2)F1 TG^+ and TG^- mice were cultured with soluble anti-Lyb-2 mAb (18). In two separate experiments, the mAb at 10 μg/ml stimulated both TG^+ and TG^- spleen cells to proliferate, and when the response per B cell number is considered, anti-Lyb-2 stimulated the TG^+ somewhat better than the TG^- cells. In one representative experiment, TG^+ spleen cell cultures (39% B cells) responded with 11,300 cpm/culture (medium control - 1,670 cpm/culture), and TG^- spleen cells cultures (60% B cells) responded with 10,400 cpm/culture (medium control - 1,510 cpm/culture). Since this allo-specific mAb stimulates B cells through a

membrane receptor separate from the IgM receptor, these data demonstrate that the defect in the TG^+ B cells is limited to the membrane IgM receptor.

Addition of Il-4 does not rescue the anti-μ responce of Anti-PC B cells. Interleukin 4 (IL-4) has been shown to function as a viability factor for B cells (21) and to synergize with suboptimum doses of anti-μ in the induction of B cell poliferation (22). The data in Figure 2 show that the addition of IL-4 to anti-μ stimulated B cells has no effect on the TG^+ B cells, whereas, the TG^- B cells exhibit an 8 fold higher response to anti-μ plus IL-4 than to anti-μ alone.

Anti-μ-induced killing of B cells from 207-4 transgenic mice. From the above data, the unresponsiveness of TG^+ B cells to soluble anti-μ appears to be due to some inherent property of the B cells rather than a result of external mediators. It has been shown that anti-μ treatment of neonatal B cells, which are predominantly sIgM-only (23,24) results in down modulation and failure to reexpress sIgM receptors (13), whereas, mature sIgM:sIgD B cells will reexpress these receptors within 18 hr of anti-Ig stripping (13). It was therefore possible that soluble anti-μ was causing a similar receptor down modulation on the IgM-only TG^+ B cells; thus, no response would be seen because multiple rounds of anti-μ signaling are required to get effective induction of proliferation (1). Alternatively, it was possible that the anti-μ stimulation of TG^+ B cells resulted in death of the cells rather than proliferation. To test both of these possibilities, TG^+ and TG^- spleen cells were incubated with soluble goat anti-μ antibody, control goat IgG, or medium alone for one hr at 37°C to allow binding and capping of the sIgM on the B cells. The cell suspensions were then washed, and a portion of the cells was stained with biotin-conjugated anti-B220 antibody plus either FITC-conjugated

goat anti-μ, anti-μ^a, or rabbit anti-goat-IgG. Only low levels of goat antibody remained after the stripping and wash procedure (data not shown). The remainder of the cells were incubated overnight in RPMI-1640 + 10% FCS to allow regeneration of the membrane IgM and were then stained for B220, IgM and IgM^a-allotype. The results of three experiments are shown in Table VI. The TG^+ and TG^- spleen cell populations averaged 18 and 30.0% B cells, respectively. After 1 h incubation with soluble anti-μ, staining of sIgM was reduced to less than 1% in the TG^+ and TG^- cultures; thus, sIgM was efficiently capped and removed from both cell types. In both the TG^+ and TG^- populations, the loss of $B220^+IgM^+$ cells was balanced by an increase in the number of $B220^+IgM^-$ cells. Twenty-four hrs. after treatment with goat anti-μ, all (44%) of the TG^- splenic B cells had reexpressed sIgM. In marked contrast, only 7% of the TG^+ B cells had reexpressed their surface IgM and very few $B220^+IgM^-$ cells remained in the culture. In as much as 20% TG^+ B cells remain the medium control, these results indicated that approximately 2/3 of the TG^+ B cells were induced to die within the first 24 hr. following anti-μ stimulation. The B cells that remained did not appear to down modulate their receptors. When the same experiment was conducted with purified B cells, the TG^+ cultures went from 80% B cells at time 0 to 49% 24 hr. later, whereas, the TG^- cultures went from 83% to 74% B cells in the same time period. These findings suggest that the failure of TG^+ B cells to proliferate following anti-μ stimulation could be due to preferential killing of these sIgM-only B cells; however, these receptor-regrowth experiment do not duplicate the culture condition used in the proliferation studies described above where the anti-μ antibodies remained in the culture throughout the 48 hr. culture period. Bulk cultures of purified TG^+ and TG^- B cells were

therefore incubated continuously with anti-μ and samples removed and analyzed at 4, 24, and 48 hrs. The data in Figure 3 show that under conditions of continuous anti-μ stimulation there is no difference in the rate of cell death in TG^+ and TG^- B cells; however, the slope of the regression lines was always slighly greater in the presence of anti-μ. These results suggest that induction of apoptosis by anti-μ cannot account for the lack of a proliferative response in the 207-4 TG^+ B cells.

Proliferation of $\mu\kappa$ anti-TNP transgenic splenic B cells following stimulation with soluble anti-μ. The restricted anti-μ stimulation defect observed in the B cells from 207-4 transgenic mice could be the result of the following possibilities. 1) The B cells may be arrested at a stage of development which is easily tolerizable, i.e. $sIgM^+IgD^-$. 2) The B cells may be unresponsive due to a previous encounter with autologous or environmental PC, which has resulted in a selective anergy or the programing of selective activation pathways. Other investigators have demonstrated that the B cells expressing endogenous IgM in M54 and Sp6 transgenic mice appear to be selected and can exhibit characteristics of sIg activated cells (25-27). 3) There may be an activation defect common to all $\mu\kappa$ transgenic mice, which results from transgene-induced alterations in B-cell development. This latter possibility was addressed by stimulating spleen cells from Sp6 anti-TNP $\mu\kappa$ transgenic mice (14) with soluble anti-μ. The data in Table VII show that both soluble anti-μ and anti-μ-conjugated Sepharose beads induce significant proliferation in both TG^+ and TG^- B-cell populations; thus, the defect in B cells from 207-4 mice is not simply the result of $\mu\kappa$ transgene expression but may be related to either the site of the transgene integration or to the specificity of the B cells expressed in these mice. However, it is also possible that the anti-PC B

cells in 207-4 transgenic mice arose from a different subset of B cells than the anti-TNP B cells in Sp6 transgenic mice, or that the anti-PC-specific B cells have been arrested at a early stage in their development.

Analysis of 207-4 B cells for Ly-1 and Mac-1 cell surface markers. Lamers et al. (15) have shown that Sp6 transgenic mice have low numbers of Ly-1 (CD5) B cells in their spleen and peritoneal cavity, whereas, Herzenberg et al. (28) found that virtually all the B cells in M54 μ-only transgenic mice were of the B-1 phenotype (29). To further elucidate the nature of 207-4 TG^+ B cells, the frequency of Ly-1 expression in the 207-4 anti-PC transgenic B cell population was examined by staining spleen and peritoneal cells from TG^+ and TG^- littermates with FITC-conjugated polyvalent and monoclonal anti-IgM and biotin-conjugated anti-Ly-1 or anti-Mac-1 antibodies. Because of the mouse-to-mouse variability in the cells from TG^+ mice, Table VIII includes the results obtained from 4 individual TG^+ mice. Less than 3% $Ly\text{-}1^+$ or $Mac\text{-}1^+$ B cells were observed in the spleens of either TG^+ or TG^- mice. Similar to the M54 transgenic mice analyzed by Stall et al. (30), the majority of peritoneal B cells from 207-4 anti-PC TG^+ mice expressed both the transgene encoded μ^a-allotype and the endogenous μ^b-allotype on their surface; however, unlike the M54 mice, less than 35% of the 207-4 peritoneal B cells express Ly-1, while 41 to 73% of these B cells express Mac-1. In the TG^- controls, Ly-1 and Mac-1 were observed on approximately 25% and 39% of the peritoneal B cell, respectively.

DISCUSSION

The induction of proliferation of mouse B lymphocytes in vitro by anti-Ig antibodies has been demonstrated in numerous studies to be a valuable tool for analyzing the ligand-receptor events associated with activation of B lymphocytes through their membrane IgM and IgD receptors. Activation of mouse B lymphocytes by anti-μ has been shown to be a function of a mature subset of Lyb-5^+ B cells (1). The data presented in this manuscript show that the M167 $\mu\kappa$ transgenic mouse line, 207-4 Bri, has a defect in its B lymphocytes in that they can not be stimulated to proliferate in vitro through their membrane IgM receptor by soluble anti-μ antibodies. This unresponsiveness was demonstrated to be independent of the dose of anti-μ antibody used or the time of assay of the cultures, and was not overcome by co-stimulation with IL-4. Furthermore, depletion of T cells from the cultures by treatment with anti-Thy1.2 plus C' was unable to reverse this unresponsiveness (Table II), and co-culture of TG^+ cells with TG^- cells did not induce any suppression in the TG^- cells in the same culture. The unresponsiveness was therefore not due to active T-cell suppression or any suppressive cytokine produced in the culture.

An unresponsiveness or diminished responsiveness of mouse B cells to soluble anti-Ig has been previously noted for 1) B cells of mice less than 8 weeks of age (31), 2) CBA/N splenic B cells (31,32), 3) B cell cultures stimulated with rabbit anti-Ig antibody capable of suppressing the B cells through their Fc receptors (3), and 4) B cells which have been preactivated in vivo (33-35). The unresponsiveness to anti-μ in the M167 $\mu\kappa$ transgenic mice, however, appears to be independent of the age of the animal (Fig.1), and blocking of the FcR with the monoclonal antibody 2.4G2 (20) did not overcome this unresponsiveness (Table II). Furthermore, $F(ab')_2$ preparations of goat

anti-μ were not stimulatory for the B cells from these mice (Table III). These results demonstrated that unresponsiveness to anti-μ was not due to FcR inhibition.

Although the B cells from 207-4 mice were unresponsive to soluble anti-μ, they could be stimulated by anti-μ, anti-Id, anti-allotype, or PC insolublized on Sepharose beads (Tables I & IV). A similar dichotomy between unresponsiveness to soluble anti-μ and responsiveness to insolublized anti-μ also occurs with Lyb-5$^-$ B cells from xid mice (32) and in neonatal B cells (36). Although Lyb-5$^-$ B cells do not proliferate in response to anti-μ treatment, they have been shown to increase in size (37) and to increase their membrane-associated Ia antigen (38). Similar to xid B cells, the 207-4 B cells also increase in size and increase their surface Ia (G. Azzolina and D.S. Sieckmann, unpublished data); however, these M167-Id$^+$, PC-specific B cells are clonally deleted in xid mice (11) and therefore, they are not equivalent to Lyb-5$^-$ B cells. The spleen cells from 207-4 mice also lack both the Ly-1 and Mac-1 surface markers characteristic of CD5$^+$ B cells, although substantial numbers of CD5$^+$ B cells occur in their peritoneal cavity (Table VIII). B cells from 207-4 mice resemble B cells from neonatal mice (23,24) and B cells which have recently emerged from the bone marrow of adult mice (12), in that they express high levels of sIgM and they lack sIgD, and thus, they could represent immature B cells. However, we have not been able to demonstrate the presence of the BP-3 alloantigen (12) on their spleen cells (unpublished data), which suggests that they may not be equivalent to either of these types of B cells.

From the data presented in Table VI, it appears that a 1 hr pulse of soluble anti-μ leads to death of many TG$^+$ B cells. However, when TG$^+$ and TG$^-$

B cells were continuously stimulated with anti-μ antibodies, there was no difference in their death rates. Thus, the failure of 207-4 TG^+B cells to proliferate following anti-μ stimulation cannot be accounted for by induction of apoptosis in these cells. Nunez (unpublished data) has however observed a reduction in the size of many of the TG^+ anti-μ-treated B cells by flow cytometry. Furthermore, introduction of the BCL-2 oncogene into the 207-4 mice allows some of the TG^+ B cells to proliferate following stimulation with anti-μ.

Three alternate hypotheses could explain the restricted anti-μ stimulation defect observed in the B cells from 207-4 transgenic mice; 1) these B cells could be arrested at an immature stage of development i.e. $sIgM^+IgD^-$; 2) this defect could be the consequence of a previous encounter with autologous or environmental PC which has resulted in a restricting of the biochemical activation pathways which can be utilized on subsequent encounters with ligand; or, 3) it could be an activation defect in these M167 $\mu\kappa$ transgenic mice, which results from transgene-induced alteration in B cell development. This defect is not common to all B cells from $\mu\kappa$ transgenic mice, since spleen cells from the $\mu\kappa$ anti-TNP Sp6 transgenic mice proliferate in response to both soluble anti-μ and anti-μ-conjugated to Sepharose beads (Table VII). However, it is known that the TG^+ anti-TNP Sp6 transgenic B cells, that co-express endogenous sIgM, also express sIgD (14,15). This suggests that the B cells in the TNP-transgenic mice may have fully matured, whereas, those in the M167 PC-specific transgenic mice may be immature. Of these alternate hypotheses, we favor the second. We have recently shown (9) that PC-specific cells are positively selected and expanded during their early development via a receptor-mediated process which appears to be antigen-

driven. Inasmuch as PC is ubiquitous, i.e. it is present on the surface of both gram positive and gram negative bacteria, in the food and bedding of the mice, and also present in autologous membranes, it appears likely that some type of thymus-independent event may be responsible for this selection. Hornbeck et al. (39) have recently demonstrated that the M167-Id$^+$ B cells from the 207-4 transgenic mice exhibit a 5-fold higher level of phosphomyristin C than the B cells from their TG$^-$ littermates. Phosphomyristin C is a principle phosphorylation substrate for protein kinase C, and it is induced in B cells following cross-linking of their sIgM receptors (40). The fact that this sIgM receptor-inducible substrate is already elevated to levels seen in anti-μ-stimulated TG$^-$ B cells suggests that the TG$^+$ B cells have previously undergone receptor-mediated signalling and may be anergic to soluble anti-μ cross-linking signals in the absence of appropriate cognate T-cell help. Anti-μ-Sepharose may result in proliferation rather than cell death due to signaling through alternate biochemical pathways as suggested by Brunswick et al. (41-43). We (6) and others (10) have shown that these M167-Id$^+$ B cells appear to respond normally in vivo and in vitro to thymus-dependent forms of PC; however, Pinkert et al. (44) found that only 1 in every 10^3 B cells from these 207-4 transgenic mice would respond in the splenic fragment assay. A. Abbas and L. Chang (personel communication) have demonstrated that activated T cells are capable of preventing anti-μ induced death in neotatal B cells; thus, the intervention of T cells via CD40 may be the normal physiological event which prevents antigen-activated B cells from subsequent death.

The B cells from these M167 $\mu\kappa$ anti-PC transgenic mice present a unique opportunity for elucidating the difference(s) in the biochemical pathway(s) that lead either to cell proliferation or cell death following signal

transduction through the same Ig-receptor. High concentrations of soluble anti-μ have been shown to induce both intracellular and extracellular calcium transport and increased phosphotidylinositol (PI) turnover within minutes of sIgM receptor cross-linking (45). We have analyzed PI turnover in the B cells from these mice and demonstrated that this early activation event was not altered in these cells, i.e. there was no difference in PI turnover in TG^+ vs TG^- B cells following activation with anti-μ, LPS, or aluminum fluoride (46). Preliminary analysis of Ca^{+2}-flux in these cells also indicates that this activation step is unaltered, although initial unstimulated Ca^{+2} levels may be higher in the B cells from TG^+ mice (J. Mond, personal communication). These data may indicate that the initial biochemical pathways that ultimately lead to either the induction of proliferation or cell death following high dose anti-μ cross-linking of sIgM receptors are shared. Elucidating where these pathways diverge and distinguishing what determines which pathway will be utilized should lead to new insights on B cell development and regulation.

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TABLE I

In vivo stimulation of 207-4 $\mu\kappa$ transgenic spleen cells with anti-Ig antibodies[a]

Mitogen	Transgene Positive	Transgene Negative	BALB/cByJ	C57BL/6J
	CPM per Culture X 10^{-3} (± SEM)[b]			
Medium	5.2 (±0.7)	2.6 (±0.4)	6.2 (±0.6)	5.1 (±0.1)
Goat anti-μ	6.9 (±0.6)	59.7 (±3.2)	93.0 (±4.2)	114.0 (±5.1)
Goat anti-μ-Seph.	79.2 (±1.2)	96.0 (±1.9)	162.0 (±6.3)	127.0 (±2.3)
Anti-μ^a-Seph.	51.4 (±2.0)	2.6 (±0.3)	71.5 (±3.6)	5.0 (±0.2)
Anti-μ^b-Seph.	5.4 (±0.2)	35.3 (±3.9)	5.3 (±0.2)	37.3 (±3.3)
Goat anti-κ-Seph.	87.4 (±4.1)	102.0 (±0.4)	177.0 (±0.7)	118.0 (±5.0)
LPS	52.9 (±1.8)	54.5 (±0.5)	62.7 (±1.4)	106.0 (±1.5)

[a] Spleen cells from individual TG^+ and TG^- 207-4 mice were cultured in the presence of soluble goat anti-μ (100 μg/ml), goat anti-μ-Sepharose (1:150), goat anti-κ-Sepharose (1:100) anti-μ^a-Sepharose (DS1-Sepharose, 1:100), anti-μ^b-Sepharose (AF6-78.25-Sepharose, 1:100) as described in MATERIALS AND METHODS.

[b] Data represent the geometric mean of triplicate cultures rounded off to two or three significant figures.

TABLE II

Stimulation of anti-thy 1.2 plus C' treated transgene positive and transgene negative B cells from young and old mice[a]

Stimulating Agent	Transgene Positive		Transgene Negative	
	Young	Old	Young	Old
	CPM per Culture x 10^{-3} (± SEM)[b]			
Media	0.4 (±0)	0.5 (±0)	1.5 (±0)	2.4 (±0)
Goat anti-μ	1.6 (±0.1)	2.0 (±0)	56.3 (±0.9)	93.5 (±10.1)
Goat anti-μ-Seph.	27.3 (±1.4)	14.3 (±0.4)	93.9 (±8.3)	77.4 (±4.0)
Anti-V_H1-Id-Seph.	267.0 (±32.7)	382.0 (±14.6)	1.9 (±0.1)	3.0 (±0.1)
Anti-M167-Id-Seph.	155.0 (±5.2)	209.0 (±14.5)	1.8 (±0.1)	2.3 (±0.1)
PC-Seph.	203.0 (±13.0)	487.0 (±67.6)	1.4 (±0.1)	2.1 (±0.1)
Rat-IgG-Seph.	0.5 (±0.1)	1.0 (±0)	1.7 (±0.1)	2.3 (±0.2)
LPS	32.5 (±2.4)	25.1 (±0.9)	56.1 (±1.9)	51.5 (±3.0)
Con A[c]	2.5 (±0.1)	0.7 (±0)	5.6 (±0.5)	1.7 (±0.2)

[a] Spleen cells (10^7/ml) from old (16 mo) and young (4 mo) mice were treated with anti-Thy1.2 plus C'. Following treatment, all groups of cells were greater than 80% B cells as determined by flow cytometry. Cells were then cultured and assayed as described in Table I.

[b] Data represent the geometric mean of triplicate cultures rounded off to two or three significant figures. This experiment was performed three times with similar results.

[c] Prior to anti-Thy 1.2 and C' treatment, all groups showed in excess of 175,000 cpm following stimulation with Con A.

TABLE III

Anti-μ stimulation of transgene positive and transgene negative spleen cells in the presence of anti-Fc receptor antibody[a]

Mitogens	Concentration of Anti-FcR (μg/ml)	C57BL/6[b]	Transgene Positive	Transgene Negative
		CPM per culture x 10^{-3} (± SEM)		
Medium	Nil	4.5 (±0.1)	0.4 (±0.2)	1.4 (±0.1)
	125	5.5 (±0.5)	0.2 (±0)	1.5 (±0.3)
	250	7.8 (±0.2)	0.3 (±0.1)	3.7 (±0.5)
Goat anti-μ	Nil	86.2 (±1.1)	0.9 (±0.1)	20.1 (±2.9)
	125	97.6 (±3.1)	0.9 (±0)	63.9 (±1.7)
	250	86.5 (±1.9)	0.8 (±0.1)	67.4 (±5.7)
Goat anti-μ-Seph.	Nil	129.0 (±9.7)	15.6 (±2.5)	63.6 (±3.2)
LPS	Nil	76.4 (±3.9)	24.3 (±2.4)	61.3 (±2.4)

[a] Spleen cells (3 x 10^5) of individual TG^+ and TG^- mice were cultured in triplicate wells in the presence of soluble goat anti-μ (100 μg/ml) and 2.4G2 monoclonal anti-FcR antibody, or Sepharose conjugated goat anti-μ (1:200) or LPS (50 μg/ml) for 48 h prior to pulsing with ^{3}H-TdR for 16 h. Results are represented as a geometric mean of the CPM/culture rounded off to two or three significant figures.

[b] This experiment was performed three times with similar results. In two other experiments, treatment of C57BL/6 spleen cells with 125 μg/ml 2.4G2 antibody in the presense of goat anti-μ, increased the proliferative response from 43,700 cpm to 86,800 cpm (exp. 1) and from 86,200 cpm to 97,600 cpm (exp. 2).

TABLE IV

$F(ab')_2$ anti-μ stimulation of transgene positive and transgene negative spleen cells[a]

Stimulating Agent	Concentration (μg/ml)	Transgene Positive	Transgene Negative
Media		3.6 ($\pm$0.2)	5.4 ($\pm$0.4)
LPS		43.8 ($\pm$3.4)	88.4 ($\pm$4.9)
Goat anti-μ-Seph.		39.5 ($\pm$1.0)	77.7 ($\pm$3.5)
Goat anti-μ	100	6.1 ($\pm$0.4)	69.2 ($\pm$3.8)
	50	5.8 ($\pm$0.7)	52.9 ($\pm$2.9)
	10	2.8 ($\pm$0.4)	17.3 ($\pm$0.4)
	5	3.8 ($\pm$1.1)	5.4 ($\pm$1.4)
	1	2.8 ($\pm$0.4)	2.0 ($\pm$0.1)
$F(ab')_2$ anti-μ	61	12.4 ($\pm$0.7)	96.1 ($\pm$3.2)
	31	8.0 ($\pm$0.2)	69.7 ($\pm$9.8)
	6	5.5 ($\pm$0.2)	23.3 ($\pm$1.2)
	3	3.1 ($\pm$0.2)	7.2 ($\pm$0.3)
	0.6	3.0 ($\pm$0.3)	2.5 ($\pm$0.2)

[a] Spleen cells were cultured and data analyzed as described in Table I. Results are represented as a geometric mean of the CPM per culture x 10^{-3} rounded off to two or three significant figures $\pm$ SEM. This experiment was performed two times with similar results.

TABLE V

Spleen cells from M167 $\mu\kappa$ transgenic mice do not suppress Anti-μ induced proliferation of normal spleen cells from transgene negative littermates

Cell Source[a] (x 10^{-5})				Stimulating Agent			
Normal		Irradiated[b]		Media	Anti-μ	Anti-μ-Seph.	LPS
TG^+	TG^-	TG^+	TG^-				
3	-	-	-	2.1 (±0.1)	3.5 (±0.1)	56.7 (±0.4)	73.1 (±2.4)
-	3	-	-	2.9 (±0.1)	56.7 (±1.5)	135.0 (±3.6)	92.7 (±1.1)
1.5	1.5	-	-	3.6 (±0.2)	31.3 (±0.8)	101.0 (±7.1)	78.6 (±1.4)
-	1.5	1.5	-	1.4 (±0.1)	23.1 (±0.5)	82.3 (±5.8)	55.3 (±1.5)
-	1.5	-	1.5	1.2 (±0.1)	20.1 (±0.8)	75.0 (±3.3)	57.2 (±0.9)
-	-	3	-	0.1 (±0)	0.1 (±0)	0.1 (±0)	0.1 (±0)
-	-	-	3	0.1 (±0)	0.1 (±0)	0.1 (±0)	0.2 (±0)
1.5	-	-	1.5	0.8 (±0.1)	1.1 (±0)	32.9 (±0.8)	42.6 (±0.5)

[a] Spleen cells from individual TG^+ or TG^- mice were cultured individually or in mixed cultures as described in Table I. Results are represented as a geometric mean of the CPM per culture ± SEM x 10^{-3}. All data were rounded to two or three significant figures. This experiment was performed three times with similar results.

[b] Irradiated spleen cells received 3000 rad.

TABLE VI

Goat anti-μ treatment of spleen cells from transgene positive mice induces B-cell death[a]

	Treatment[b]	Percent of Total Cells[c]				
		Transgene Positive			Transgene Negative	
		μ^- B220$^+$	μ^+ B220$^+$	μ^{a+} B220$^+$	μ^- B220$^+$	μ^+ B220$^+$
Day 1	Untreated	0.9 ±0.3	18.3 ±2.7	16.7 ±2.5	2.4 ±1.1	30.7 ±3.8
	Goat IgG	1.0 ±0.2	18.0 ±4.4	15.2 ±0.1	1.4 ±0.6	31.4 ±3.0
	Goat anti-μ	16.2 ±4.4	1.3 ±1.0	2.4 ±1.8	30.0 ±2.5	3.5 ±1.9
Day 2	Untreated	1.1 ±0.4	20.2 ±3.6	16.4 ±5.2	1.5 ±0.2	44.7 ±4.5
	Goat IgG	1.1 ±0.4	17.7 ±0.6	13.1 ±3.1	1.8 ±0.1	47.1 ±5.7
	Goat anti-μ	2.3 ±0.9	7.2 ±0.8	4.6 ±1.4	3.3 ±0.3	44.2 ±6.0

[a] This experiment was performed three times and the results are represented as the arithmetic mean ± SEM for each experimental value.

[b] Spleen cells (1 x 10^7/ml) from TG$^+$ and TG$^-$ 207-4 mice were cultured in the presence of soluble goat anti-μ (100 μg/ml) for 1 h at 37°C, washed 3 times and cultured overnight at 37°C. In three experiments, cell recoveries from TG$^-$ and TG$^+$ cultures ranged from 24-42% and 27-33%, respectively. No significant difference in cell recovery was seen between anti-μ treated and untreated cultures.

[c] Spleen cells (1 x 10^6) were stained before and after anti-μ treatment with FITC-conjugated anti-μ and biotin-conjugated anti-B220 plus PE-Streptavidin and analyzed as described in MATERIALS AND METHODS.

TABLE VII

Anti-μ induced activation of spleen cells from Sp6 anti-TNP μκ transgenic mice[a]

Stimulating Agent	Transgene Positive	Transgene Negative
	CPM per culture x 10^{-3} (±SEM)	
Media	2.1 (±0.4)	4.5 (±0.2)
Anti-μ	47.3 (±4.4)	73.0 (±8.2)
Anti-μ-Sepharose	82.1 (±1.5)	130.0 (±8.9)
LPS	58.2 (±2.2)	95.8 (±3.1)
Con A	241.0 (±4.5)	255.0 (±5.6)

[a] Data represent the mean response, rounded to two or three significant figures, of spleen cells from two TG^+ and two TG^- mice cultured individually. Data were calculated as described in Table I.

TABLE VIII

Analysis of B cell phenotype in anti-PC transgenic mice[a]

Staining Agent	Transgene Positive		Transgene Negative	
	Spleen	PEC	Spleen	PEC
IgM^a:Ly-1	2.1	3.8	-	-
	3.3	19.2	-	-
	2.0	17.5	-	-
	1.3	17.2	-	-
IgM^b:Ly-1	<1.0	4.2	1.3	12.0
	1.1	23.0	1.7	8.7
	<1.0	16.1		
	1.1	10.7		
IgM^a:Mac-1	1.4	17.5	-	-
	<1.0	27.1	-	-
	<1.0	27.2	-	-
	<1.0	27.2	-	-
IgM^b:Mac-1	<1.0	17.4	<1.0	18.3
	<1.0	33.3	<1.0	13.4
	<1.0	29.1	-	-
	<1.0	35.5	-	-
Total IgM	27.6	35.7	24.5	53.5
	20.3	65.6	25.1	34.3
	13.2	58.9	-	-
	11.2	48.6	-	-

[a] Cells from the spleen and peritoneum of four TG^+ and two TG^- mice were stained and analyzed as described in MATERIALS AND METHODS. Data represent the percent positive cells in the total cell population of each individual mouse.

FIGURE LEGENDS

Figure 1. Response of individual TG^+ and TG^- mice to soluble or Sepharose conjugated goat anti-μ and the total IgM bearing B cells in spleens of TG^+ and TG^- mice. Data were compiled from 9 experiments in which 26 TG^+ and 25 TG^- spleens were cultured individually at 3×10^5 cells per well with (A) goat anti-μ (100 μg/ml) or (B) goat anti-μ Sepharose (1:100). The geometric mean of triplicate cultures is plotted against the age of the spleen donor. In C, spleen cells of TG^+ and TG^- mice were individually stained with a FITC-conjugated goat anti-μ and analyzed by flow cytometry.

Figure 2. The unresponsiveness of B cells from M167 $\mu\kappa$ transgenic mice cannot be reversed by IL-4. Purified splenic B cells were cultured with goat anti-IgM (50μg/ml) +/- IL-4 (1000 U/ml) or with LPS (50 μg/ml). Cells were pulsed with [^{3}H]thymidine for 4 hrs. prior to harvesting.

Figure 3. TG^+ and TG^- B cells die at the same rate in the presence of anti-μ antibodies . Purified TG^- (A and C) and TG^+ (B and D) B cells at 10^6/ml were cultured in T25 flasks for 48 hr. in the presence (circles) or absence (triangles) of goat anti-μ antibodies (100μg/ml). Samples were taken at the times indicated and the cells counted and stained with FITC-anti-μ and PE-anti-B220. Linear regression analysis was run on the data from two experiments. R^2 values range from 0.85 to 0.99 and the slope of the anti-μ treated cells in each panel is slightly greater than that of the media control.

Fig 1

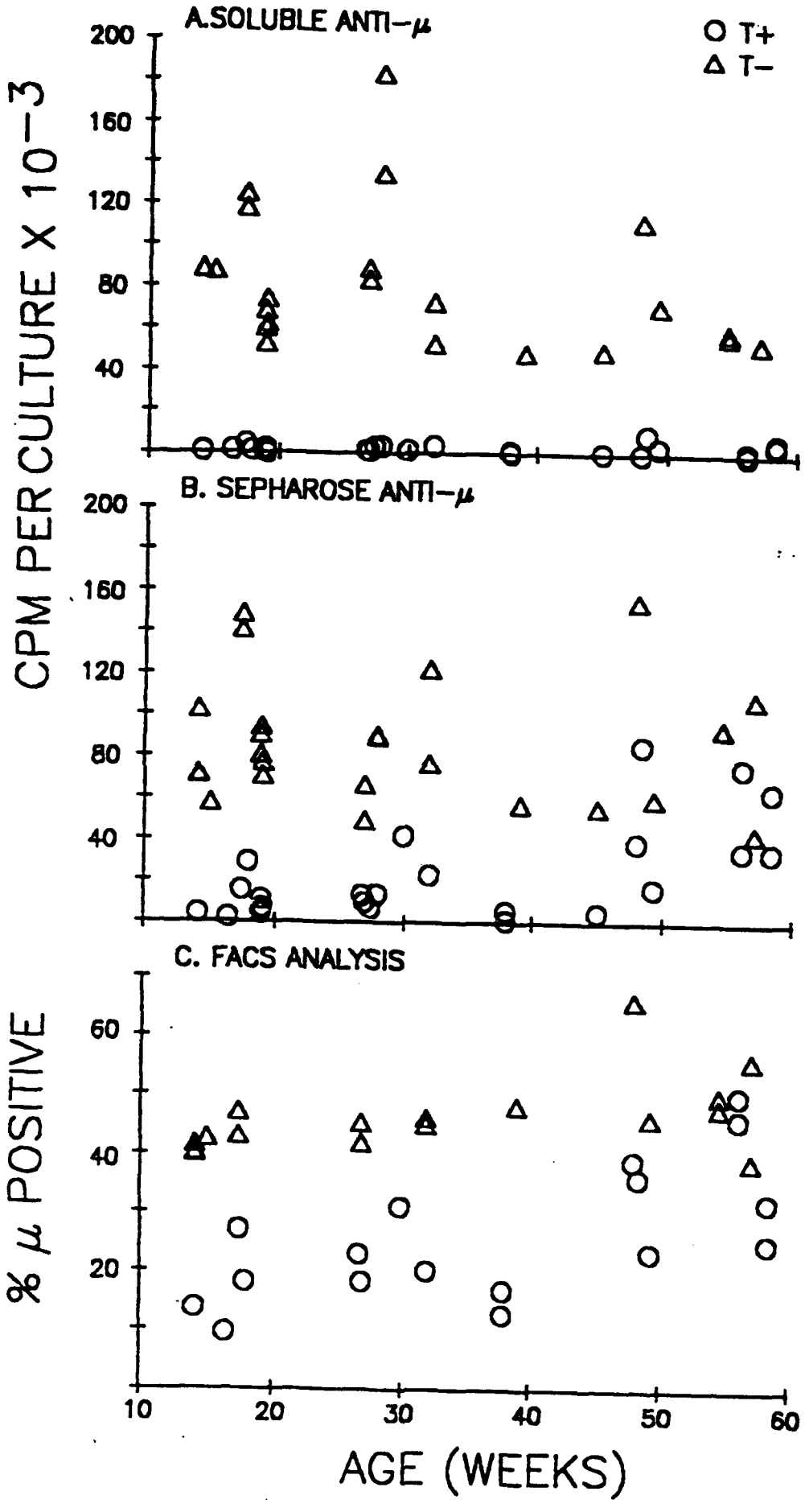
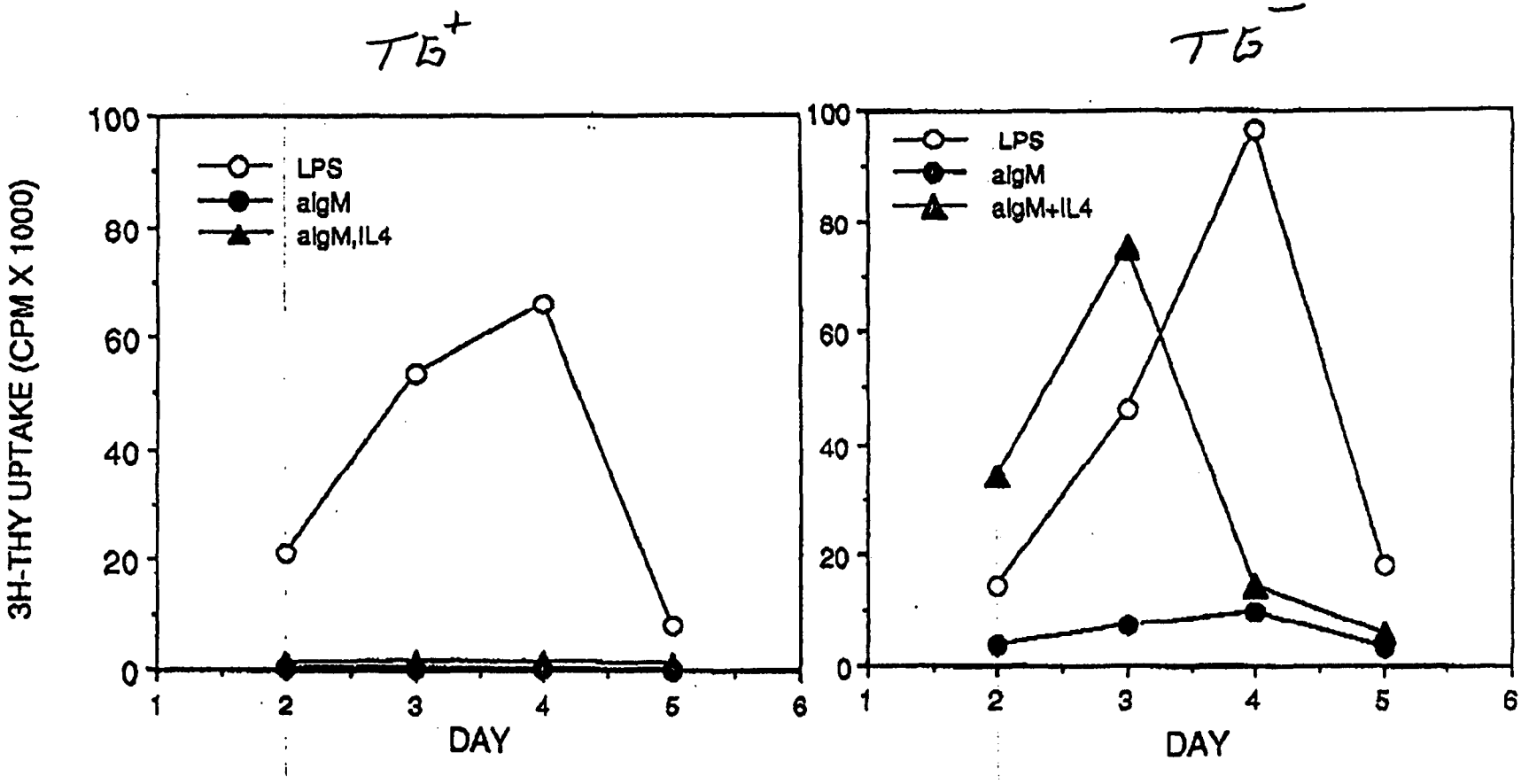
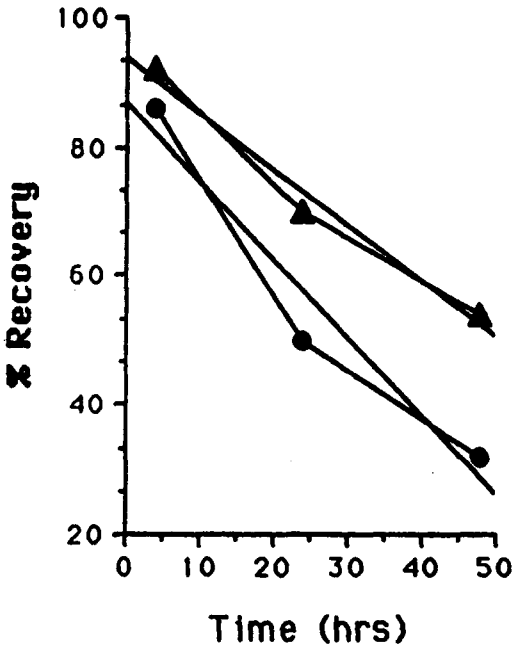
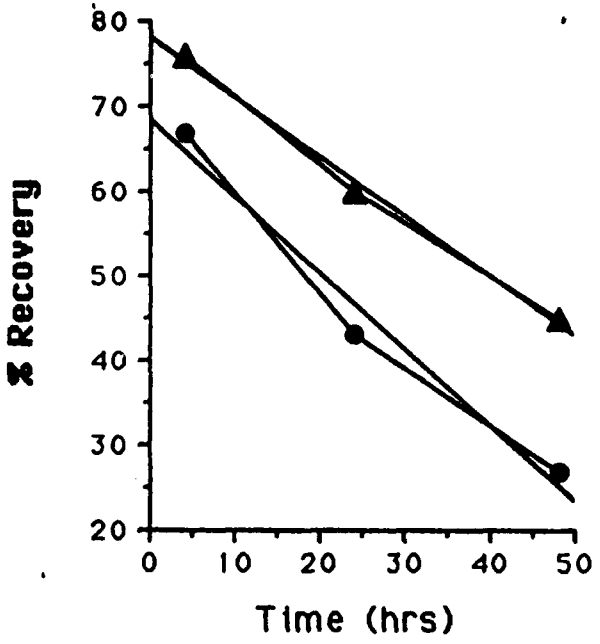

A.SOLUBLE ANTI-μ
O T+
Δ T-
B. SEPHAROSE ANTI-μ
C. FACS ANALYSIS
CPM PER CULTURE X 10-3
% μ POSITIVE
AGE (WEEKS)
200
160
120
80
40
60
20
10
30
50

Fig. 2

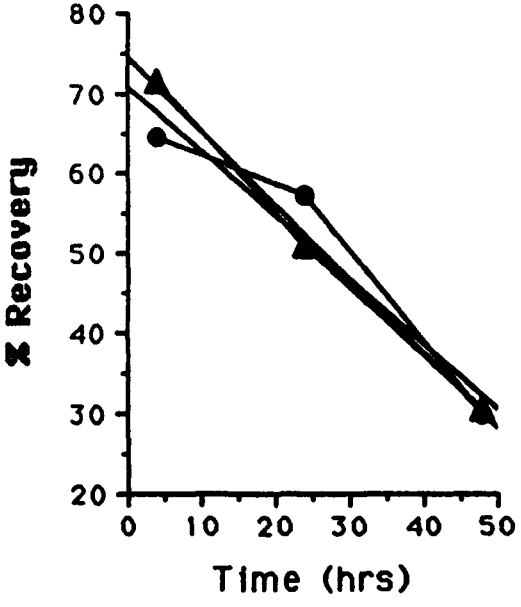

T5+
T5−
3H-THY UPTAKE (CPM X 1000)
LPS
aIgM
aIgM,IL4
aIgM+IL4
DAY
0
20
40
60
80
100
1
2
3
4
5
6


100
80
60
40
20
% Recovery
0 10 20 30 40 50
Time (hrs)

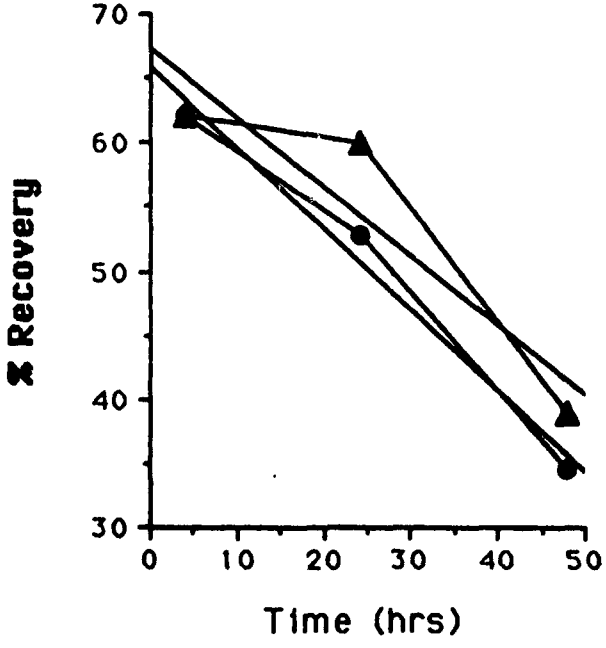
B TG+


80
70
60
50
40
30
20
% Recovery
0 10 20 30 40 50
Time (hrs)

C TG-


80
70
60
50
40
30
20
% Recovery
0 10 20 30 40 50
Time (hrs)

D TG+


70
60
50
40
30
% Recovery
0 10 20 30 40 50
Time (hrs)